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Extract Standardization in Ethyl Acetate Fraction from *Sargassum Hystrix* as Inhibitor of α-Amylase and α-Glucosidase

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ABSTRACT

Seaweed has great potential in the food and pharmaceutical fields, and it is well known for its anti-diabetic property. The standardization of the preparation of ingredients or food products is very important in the development of drugs. Therefore, this study aims to determine the results of the standardization of extracts in the ethyl acetate fraction of *Sargassum hystrix* as inhibitors of α-amylase and α-glucosidase. Furthermore, *S. hystrix* was extracted and partitioned using chloroform, ethyl acetate, and methanol. The ethyl acetate fraction was tested for standardization containing specific and non-specific parameters. The specific parameters include the identity of simplicia, organoleptics, number of compounds dissolved in certain solvents, total flavonoids, and antidiabetic properties. Meanwhile, non-specific parameters include total bacteria and mold, as well as moisture, ash, and heavy metal content (Pb and Cd). In addition, the simplicia ethyl acetate fraction was obtained from brown seaweed *S. hystrix* using all parts of the plant. The results showed an organoleptic appearance in the form of a coarse powder approaching crystals, which is dark yellow in color with reduced fish smell. The levels of the compound dissolved in water and ethanol were 71.93 ± 4.26 and 14.33 ± 0.82% respectively. The total flavonoids were 2.23 ± 0.10 mgQE/g, and spot 3 compounds obtained from TLC of ethyl acetate fraction as antidiabetic were pentadecanoic acid (2.08%), benzenedicarboxylic acid (77.77%), and hexadecanoic acid (5.14%). Furthermore, the values for the water content, ash content, total bacteria, and total mold were 9.43 ± 0.46%, 77.62 ± 0.91%, 163.33 ± 126.62 CFU/g, and 3.36 x 10^3 ± 1.8 x 10^3 CFU/g, respectively, while the number of Cd and Pb were not obtained.

Keywords: Antidiabetes, extract, ethyl acetate fraction, *Sargassum hystrix*, standardization

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INTRODUCTION

Diabetes mellitus is a group of complex metabolic disorder characterized by hyperglycemia in an untreated condition[1]. This non-communicable degenerative disease was a problem for public health in Indonesia and on a global scale[2]. In 2019, 422 million people were already affected by diabetes worldwide[1] and this number is expected to increase in 2035 to 592 million.

Some of the current efforts to overcome this disease include non-pharmacological (such as weight control, diet, and exercise) and pharmacological therapies (such as insulin hormone administration and oral hypoglycemic drugs). Generally, patients prefer to participate in pharmacological therapy because of the difficulties experienced in the non-pharmacological type[3]. This pharmacological therapeutic approach inhibits the degradation of oligo and disaccharides during digestion by enzymes that hydrolyze carbohydrates in the digestive tract[4], such as α-amylase in saliva and pancreas, as well as α-glucosidase located in the first part of the small intestine[5]. However, the enzymatic actions of α-glucosidase and α-amylase are inhibited by using acarbose. This drug is administered with an initial dose of 50 mg and increased gradually to 150-160 mg/day[6], and it has side effects, which may include flatulence, weight gain[7], disturbances, malabsorption, excessive gas production, diarrhea, and dyspepsia[8]. Therefore, there is currently a search for natural products for diabetes, especially herbal ones, one of which is derived from seaweed. The secondary metabolites of seaweed have antibacterial, antiviral, and antifungal[9], as well as anti-diabetic properties[10]. *Sargassum hystrix* is a brown seaweed that contains polyphenols, which possess antidiabetic properties[11]. Furthermore, it has the highest antioxidant activity compared to other *Sargassum* seaweed species[12]. The study conducted by Samudra et al.[13] showed that *S. hystrix* has potential as an antidiabetic almost similar to acarbose. Also, Azizi et al.[14], Azizah et al.[15], and Husni et al.[16], using 3 different types of fractions including methanol, chloroform, and ethyl acetate reported that *S. hystrix* had the highest inhibitory activity in inhibiting α-glucosidase.

One of the important stages in drug development is the standardization of herbal extracts. It is a set of measurement procedures and methods, in which the result parameters are elements related to the concept of pharmaceutical quality corresponding to chemical and biological standards[17]. Herbal extracts can be presented as raw, intermediates, and finished product[18] after meeting predetermined requirements. The quality requirements consist of various specific and non-specific standard parameters established by the Ministry of Health Republic of Indonesia[19]. Furthermore, many studies related to standardization have been conducted, such as in the specific and non-specific examination of hydrotropic andrographolide extraction from sambiloto[20]. The determination of non-specific and specific standardization parameters of henna nail leaf extract[21] was also conducted. However, there are no studies on the standardization of seaweed extract, especially *S. hystrix* as an α-amylase and α-glucosidase inhibitor. Therefore, this study aims to

determine the standardization of the extract in the ethyl acetate fraction of *S. hystrix.*

MATERIALS AND METHODS

Materials

The raw materials used include *Sargassum hystrix* obtained from the Teluk Awur bay, Jepara Indonesia. Furthermore, ethanol, methanol, chloroform, ethyl acetate (Merck KgaA, Germany), acarbose (Glucobay, Bayer Pharmaceuticals, Germany), α-glucosidase type I from *Saccharomyces cerevisiae*, α-amylase type II-A from *Bacillus* sp., p-nitrophenyl-α-D-glucopyranoside (Sigma Aldrich, USA), dinitrosalycilic acid (DNS), Na_2CO_3, quercetin (Sigma Aldrich, USA), potassium acetate (Himedia, India), $AlCl_3$, Plate Count Agar (PCA) medium, Potato Dextrose Agar (PDA) medium, and acid nitrate (Merck KgaA, Germany) were used.

Sample preparation

The study was conducted in stages including collection, identification, and sample preparation. Seaweed samples were collected from Awur bay, Jepara Indonesia, and were further identified at the Plant Taxonomy Hydrobiology Laboratory, Faculty of Biology, Universitas Gadjah Mada. The sample preparation was conducted by drying the *S. hystrix* and was then blended into a powder form. Meanwhile, the extraction, partitioning, and standardization process followed immediately.

Seaweed extraction and partition

S. hystrix extraction was conducted according to the method of Yang et al.[22] modified. The principle was to perform maceration at room temperature using the ratio of 1: 8 (717 g of *S.hystrix* powder dissolved in 6,000 ml of methanol). Subsequently, it was stirred to ensure the solvent was all over the surface of the sample. The extraction process was repeated three times for 24 hours to change the solution for each replication. The solution containing the sample was filtered to reduce residue or impurities and then evaporated. Liquid-liquid partition was conducted in accordance with Yang et al.[22] with modifications. In addition, the partitions were conducted in stages, starting with nonpolar, semi-polar, and polar solvents (chloroform, ethyl acetate, and methanol). The ratio used between the sample: solvent was 1: 18.75 and between solvents of 1: 1. The *S.hystrix* extract was dissolved and stirred until it was homogeneous and put into a separating funnel to be partitioned with a ratio of chloroform and methanol (1: 1). The methanol was mixed with water in a ratio of 3: 2 and the partition seemed to be completely separated. After separating the chloroform fraction, ethyl acetate was added in a ratio of 1: 1 to obtain its fraction with methanol. The partition process was conducted by shaking the sample and solution in a separating funnel for 10 minutes until it was completely separated. Each partition was repeated three times for each repetition.

Standardization of specific parameters

Specific parameter test includes the identity of simplicia, organoleptics, levels of compounds that dissolve in certain solvents[18], total flavonoids[23], and identification of antidiabetic compounds. The simplicia identity test was conducted by including the sample and the Latin name of the sample as well as the plant part used. Furthermore, the organoleptic test includes the appearance, smell, and color of the sample. This test was conducted using the scoring method by 6 panelists from the Fish Quarantine Agency, Quality Control, and Safety of Fishery Products in Yogyakarta. The scoring test specifications include; appearance (9: crystal, 7: coarse powder, 5: fine powder, 3: viscous, 1: liquid); smell (9: very less fishy, 7: less fishy, 5: a little less fishy, 3: fishy, 1: very fishy); Color (9: very dark yellow, 7: dark yellow, 5: slightly amber, 3: light yellow, 1: light yellow).

To a certain degree, some compounds become soluble in water and ethanol. This is evident in this study, where a total of 2.5 g of the sample was displaced with 50 ml of water to test the content of water-soluble compounds or 50 ml of 95% ethanol using a clogged flask while shaking for the first 6 hours and left for 18 hours. Furthermore, the sample was filtered, and 10 ml of the filtrate was evaporated to dryness in the evaporator cup, and the residue was heated at 105°C to a fixed weight. The percentage of water and ethanol soluble compounds to the weight of the initial extract was also calculated[18].

The total flavonoid test was based on the method described by Aminah et al.[23] modified. It was conducted in two stages, namely making quercetin standard curves and determining total flavonoid levels. In making a standard quercetin curve, 25 mg of its mass was weighed and dissolved in 25 ml of methanol. The volume of the stock solution was increased to 10 ml to obtain a concentration of 100 ppm. Furthermore, several concentrations of 6, 8, 10, 12, and 14 ppm were made from this solution. A total of 1 ml of each concentration was added to 1 ml of 2% $AlCl_3$ and 120 mM potassium acetate. The samples were incubated for one hour at room temperature, and the absorption was performed using spectrophotometry with a wavelength of 435 nm. The determination of total flavonoids was conducted by a partition sample weighing as much as 15 mg and dissolved in 10 ml of methanol. Subsequently, 1 ml of the solution was pipetted against 1 ml of 2% $AlCl_3$ solution and 120 mM potassium acetate. The samples were incubated for one hour at room temperature, while the absorbance was observed using a spectrophotometer at a wavelength of 435 nm.

The testing of antidiabetic compounds was conducted using a thin-layer chromatography (TLC) method[24]. Thereafter, the Gas Chromatography Mass Spectrometry (GC-MS) test was conducted after finding the separation spot[25]. This analysis used Agilent GC-MS (6890 GC METHOD and 5973 inert MSD) equipped with a split injector and an HP-5MS column of 0.25mm x 30m x 0.25um (Agilent 19091S-433). The step taken in this test was to dissolve the sample and administer as much as 1 µL at a temperature of 300°C. Furthermore, the sample is formed into steam and then pushed by helium gas to enter the GC capillary column. However, the compound separation process only occurs provided the sample remains in the capillary column. Compound detection was conducted in MS by firing the electrons into ionized molecules and recording their fragmentation patterns. The identification of the mass components was conducted in the Wiley 10N14 database.

Standardization of nonspecific parameters

Non-specific parameters include moisture content, ash content[26], total bacteria, total mold, and heavy metal content (Pb and Cd). The moisture content (%) of seaweeds was determined by drying 2 g of samples in a thermoregulated incubator (Memert 40050ip20, Germany) at 105°C until constant weight[26]. The ash content was determined by heating the samples for 4 h in a muffle furnace (Thermo Scientific, Germany) at 500°C[26]. Furthermore, the total bacteria test was conducted by dissolving 5 g of the sample in 45 ml of Butterfield's Phosphate Buffered (BFP) solution until it was

homogeneous. A total of 1 ml of solution was taken and diluted with 9 ml of BFP to obtain the first and second dilutions. Subsequently, the diluted solution was placed in a PCA medium and incubated for two days at a temperature of ±35°C before calculating the total number of bacteria.

The total mold test was conducted by growing the mold in agar medium and incubating at 25°C for five days, and the determination was conducted using the spread plate method. Furthermore, testing of heavy metal levels was conducted using the Atomic Absorption Spectrophotometer (AAS) method. The sample was prepared first using nitric acid before adopting the AAS testing method.

RESULTS AND DISCUSSION

Standard of specific parameters

The quality requirements for extracts consist of various specific standard and non-specific standard parameters that have been established by the Indonesian[19]. Specific parameters include simplicia identity, organoleptic testing, levels of compounds that dissolve in certain solvents (water and ethanol), total flavonoids, and anti-diabetic compounds. Conversely, non-specific parameters include moisture content, ash content, total bacteria, total mold, and heavy metal content. The results of the standardization test results of the ethyl acetate *S. hystrix* partition are shown in Table 1.

Table 1: The results of the standardization test for the ethyl acetate fraction of Sargassum hystrix

Parameter		**Results**
Specific		
Simplicity identity		Name of simplicia: Ethyl acetate fraction of brown seaweed
		Latin name: *Sargassum hystrix*
		Plant parts used: All parts of the Sargassum hystrix
Organoleptic	Appearance	Coarse powder close to crystals
	Color	Dark yellow
	Odor	Very less fishy
Content of compounds that dissolve in certain solvents		
Content of water-soluble compounds (%) (w/v)		71.93 ± 4.26
Ethanol soluble compound (%) (w/v)		14.33 ± 0.82
Total flavonoids (mg QE/g)		2.23 ± 0.10
Non-specific		
Water content (%)		9.43 ± 0.46
Ash content (%)		77.62 ± 0.91
Total bacteria (CFU/gram)		163.33 ± 126.62
Total mold (10^3 CFU/gram)		3.36 ± 1.80
Heavy metal content		
Total Pb (ppm)		0.00
Total Cd (ppm)		0.00

Simplicia identity

Simplicia is a natural remedy used as an unprocessed medicine, and it aims to provide objectivity from plant names and specifications concerning the scientific classification. Furthermore, it distinguishes the plant parts used from other raw materials based on their characteristics. The simplicia identity is shown in Table 1, and the name used in this study was the ethyl acetate fraction of *S. hystrix* brown seaweed.

Organoleptic

The organoleptic test was conducted by 6 trained panelists by observing appearance, smell, and color. This was achieved by filling in the scoring test sheet from numbers 1-9. In addition, the results of organoleptic observations of *S. hystrix* extract in ethyl acetate partition is presented in Table 1. It shows that the appearance is in the form of a coarse powder close to the crystal, while the smell is less fishy with a dark yellow. However, this result differs from the standardized organoleptic results of *Anredera cordifolia* leaf extract for inhibition of α-glucosidase, which is thick and black-green and does not have a specific aromatic odor[27]. When standardization was conducted on gedi simplicia, the leaves became brownish green with a distinctive odor and a pungent taste[18].

Content of compounds dissolved in certain solvents

The parameters of the compound dissolved in a certain solvent aim to provide an initial picture of the amount to be extracted. The solvents used in this case are water and ethanol. Within the extract, the water and ethanol dissolve polar and less polar compounds, respectively[18]. The results from the water and ethanol solvent test in the ethyl acetate *S. hystrix* partition are shown in Table 1.

The results showed that the levels of water-soluble and ethanol-soluble were 71.93 ± 4.26 and 14.33 ± 0.82% (w/v), respectively. These data show the dissolved compounds in water are greater than in ethanol. In the ethyl acetate *S. hystrix* partition there are more polar compounds (dissolved in water) than ethanol. However,

this result was different from the standardization of *Anredera cordifolia* leaf extract to inhibit α-glucosidase, while the obtained percent for water-soluble compounds and ethanol were 72.14 and 69.63%, respectively[27]. Furthermore, the standardization of gedi leaf extract compounds in water and ethanol were 7.38 ± 0.22 - 8.91 ± 0.21 and 21.12 ± 0.16 - 29.44 ± 0.20% (w/w), respectively[18]. This differs from the standardization of sembung, which obtained water and ethanol soluble compound levels of 7.80 - 15.02 and 7.58 - 9.20% (w/w), respectively[28].

Levels of total flavonoids

In this study, quercetin was used as a standard to determine the total flavonoid levels. The results showed that total flavonoid levels were 2.23 ± 0.10 mg QE/g (Table 1). The yield was low when compared with the standardization of gedi leaf extract of 23.63 ± 0.06 mg/g[18]. Conversely, it is higher than the standardization of gotu kola plant extract of 0.56% (w/w)[29].

Antidiabetic compounds in the ethyl acetate fraction of S. hystrix

TLC was conducted before GC-MS analysis to separate the compounds due to the different polarity properties. It uses an aluminum plate coated with silica gel 60-F_{254} as a stationary phase. Meanwhile, the solution acts as a mobile phase and passes through the plate following the principles of capillary. The TLC results of ethyl acetate fraction visualized with UV 366 nm using hexane: ethyl acetate: formic acid (6: 4: 0.2) is shown in Figure 1. Saifudin[30] reported that a solvent is good when compounds are easily separated from the extract by showing the number of spots formed. The results showed that the eluted spots are in the middle upwards and the compounds contained are semi-polar to polar.

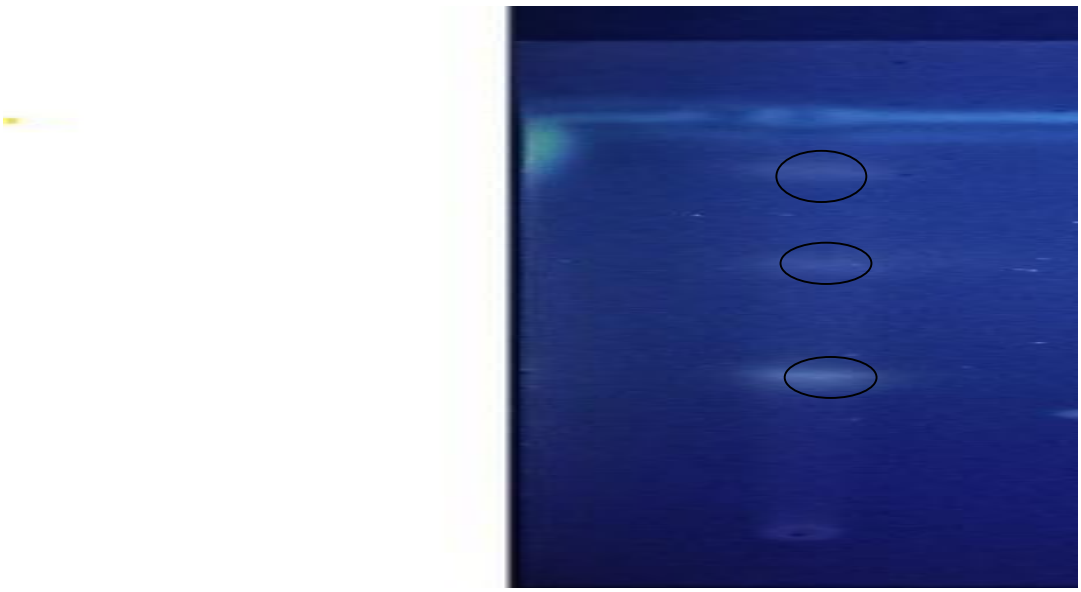

Figure 1: UV visualization of 366 nm results from TLC of ethyl acetate fraction using hexane: ethyl acetate: formic acid (6: 4: 0.2) solvent

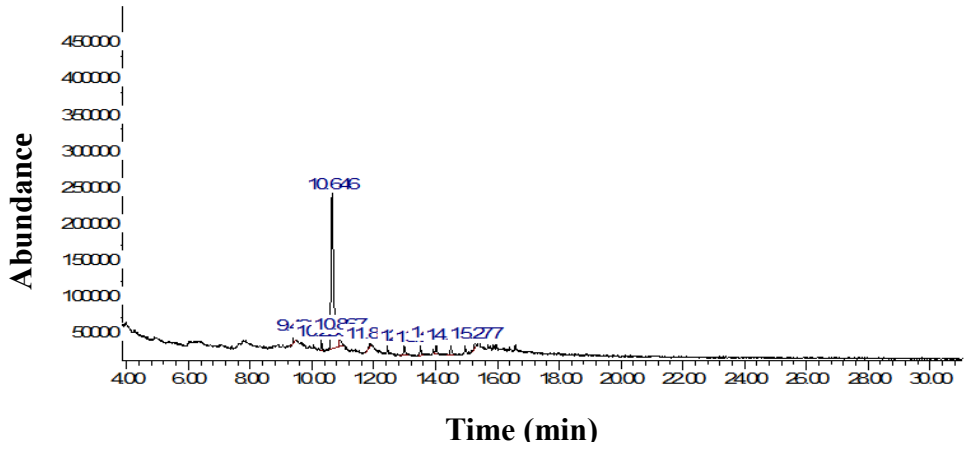

Figure 2: GC-MS chromatogram of ethyl acetate fraction of Sargassum hystrix from TLC spot number 3

Table 2: The compounds identified in the ethyl acetate fraction of Sargassum hystrix resulted from TLC spot number 3

Retention time (min)	Compounds	Area (%)	Class	Biological activity
10.297	Pentadecanoic acid	2.08	Fatty acid	Antidiabetes[35]
10.647	Benzenedicarboxylic acid/dibutyl ester	77.7	Fatty acid	Antidiabetes[31]
10.867	Hexadecanoid acid/palmitic acid	5.14	Fatty acid	Antidiabetes[34]

From the GC-MS spot 3 analysis, the results of the TLC *S. hystrix* showed that several fatty acid compounds have antidiabetic activity (Figure 2, Table 2). Among these compounds, benzenedicarboxylic acid or dibutyl ester is the most dominant, and it has enzyme inhibitory activity either in vitro or in vivo in Wistar rats[31]. Fatty acid compounds bind to the active site of the substrate to prevent the reaction with the enzyme[32]. Furthermore, it has double bonds that play a binding role in the enzyme. The greater the double bond, the higher the inhibitory activity[33]. Hexadecanoic or palmitic acid has anti-inflammatory and antidiabetic activity[34]. Pentadecanoic acid (C15: 0) has activity similar to antidiabetic type 2[35].

Non-specific parameters

Water content (%)

The determination of water content provides a minimum limit or range of the amount of water content in the material. To reduce biological activity during storage, it is easier to grow fungi and molds with higher water content. Furthermore, the duration of drying defines moisture content using air. This air-drying method is conducted until the moisture content is less than 10%. Drying aims to reduce the water content in *Sargassum* sp. because the quality is better with a reduced solvent level. However, the longer the drying time, the smaller the moisture content in the partition. Inappropriate drying process leads to changes in shape, appearance, and quality characteristics. Furthermore, lengthy drying time causes mold and rot, especially when conducted during the rainy season[36].

The results of the water content test obtained a moisture level of 9.43% ± 0.46% (Table 1) and was supported by the study on the standardization of *Anredera cordifolia* leaf extract to inhibit α-glucosidase by 8.47%[27]. In addition, 8.25 ± 2.51%[18], and 4,43%[29] were obtained on the standardization of gedi leaves and gotu kola, respectively[29]. The regulation of the head of BPOM Republic of Indonesia number 12 of 2014 reported that the requirements for the quality of water content in the form of simplicia were ≤ 10. Therefore, it is reasonable to conclude that the water content of the ethyl acetate *S. hystrix* partition extract is consistent with the standard, and it is influenced by the drying technique. Drying was conducted by air-drying to obtain standards' compliant results.

Ash content

Ash and mineral contents of material are related. Ash content determines the internal and external mineral from the initial to the end of the process. In contrast, the mineral content is in the form of organic and inorganic salts. Generally, the ash content is influenced by the halogen components (Br and I) contained in mineral salts and depends on the initial washing process before extraction[37]. Brown algae include materials with high minerals such as Na, Ca, K, Cl, Mg, Fe, and S. The components from seaweed depend on the type, age, and hydrological as well as hydro-chemical conditions of the seaweed's environment[38].

The ash content of the extract in the ethyl acetate S. hystrix partition was obtained with 77.62% ± 0.91% (Table 1). Furthermore, the results were relatively high when compared to the standardization study of *Anredera cordifolia* leaf extract to inhibit the α-glucosidase enzyme at 1.22%[27], 10.33% in sembung leaf simplicity[28], and 22 ± 1.46% in gedi leaf extract[18]. Suryani et al.[39] reported that the total mineral and ash contents in food are the inorganic residue produced after the organic materials are exhausted. The mineral and the ash contents are directly proportional. Therefore, the Increase or decrease of mineral content has the same effect on that of the ash. The amount of ash content depends on the type of seaweed and the mineral salt on its surface or the thallus.

Total bacteria

The test for the total number of bacteria was used to calculate the presence in the ethyl acetate fraction of *S. hystrix*. The results showed that the total bacteria were 163.33 ± 126.62 CFU/g (Table 1). Furthermore, the BPOM RI regulation number 12 of 2014 showed that the quality requirements for total bacteria or plate numbers on the drug were ≤ 10^6 CFU/g. Therefore, the total bacteria ethyl acetate fraction of *S. hystrix* was consistent with the requirements. These results are better than the standardization study of *Anredera cordifolia*, which was 0.45 x 10^3 CFU/g[27], and that of gedi leaf extract at 6.70 x 10^5 CFU/g[18].

Total mold

The total mold obtained in the ethyl acetate fraction of *S. hystrix* was 3.36 x 10^3 ± 1.80 x 10^3 CFU/g (Table 1). The results were quite high when compared to the *Anredera cordifolia* standardization of 0.10 x 10^3 CFU/g[27], and that of gedi leaf extract at 6.70 x 10^2 CFU/g[18]. However, the BPOM RI regulation number 12 of 2014 showed that the quality requirement for the total mold rate in drugs was ≤10^4 CFU/g. Therefore, the total mold ethyl acetate fraction of *S. hystrix* is consistent with the requirements of the BPOM RI.

Heavy metal content

Analysis of heavy metal content includes Pb and Cd. The analysis of the ethyl acetate fraction of *S. hystrix* did not obtain Pb (Table 1). However, this result is better when compared to the study of Djamil et al.[27], where the standardization of *Anredera cordifolia* has a Pb level of 0.23 mg/kg, gedi leaves of 8.00 ± 3.00 mg/kg[18], and gotu kola leaves of 1.61 ppm[29]. These results followed the quality requirements in the 2014 BPOM RI regulations which were ≤10 mg/kg. Similarly, the analysis of the ethyl acetate fraction of *S. hystrix* did not obtain Cd. In addition, the study of Djamil et al.[27] obtained standardization of *Anredera cordifolia* with a cadmium level of 0.0024 mg/kg and 0.069 ppm for gotu kola[29]. The Cd level of the ethyl acetate fraction was consistent with the requirements of the 2014 BPOM RI regulations of ≤ 0.30 mg/kg.

CONCLUSION

The simplicia identity was obtained from the ethyl acetate fraction of Sargassum hystrix brown seaweed using all plant parts. The organoleptic results showed the appearance of a coarse powder close to the crystal, dark yellow in color, and with a reduced fishy smell. Furthermore, the levels of the compound dissolved in

water and ethanol were 71.93 ± 4.26% and 14.33 ± 0.82% respectively. The total flavonoids were 2.231 ± 0.10 mgQE / g, and the compounds obtained in spot 3 of the TLC from the ethyl acetate fraction of S.hystrix were pentadecanoic (2.08%), benzenedicarboxylic (77.77%), and hexadecanoic acid (5.14%). The ethyl acetate fraction of S. hystrix has a moisture content, ash content, total bacteria and mold of 9.43 ± 0.46%, 77.62 ± 0.91%, 163.33 ± 126.62 colonies/gram, 3.36 x 103 ± 1.8 x 103 colonies/gram respectively, while Cd and Pb were not obtained.

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